AD_____

Award Number: DAMD17-03-1-0586

TITLE: Role of Rad51-Mediated Interactions in Recombination

PRINCIPAL INVESTIGATOR: Lumir Krejci, Ph.D.

CONTRACTING ORGANIZATION: Yale University
New Haven, Connecticut 06520-8047

REPORT DATE: August 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2004	Annual Summary (1 Aug 2003 - 31 Jul 2004)

4. TITLE AND SUBTITLE
Role of Rad51-Mediated Interactions in Recombination

5. FUNDING NUMBERS
DAMD17-03-1-0586

6. AUTHOR(S)
Lumir Krejci, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University
New Haven, Connecticut 06520-8047

E-Mail: Lumir.Krejci@yale.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The repair of DNA double-stranded breaks by homologous recombination is mediated by genes of *RAD52* epistasis group. Rad51 protein, a key member of the *RAD52* group, forms a helical filament on single-stranded DNA that is derived from double-stranded breaks. The Rad51-DNA filament searches for a DNA homologue, either the sister chromatid or homologous chromosome, and catalyzes the formation of a DNA joint with the homologue. Rad51 interacts with other *RAD52* group proteins including Rad52, Rad54, and XRCC3. In addition, the breast tumor suppressor BRCA2 associates with Rad51. Consistent with this, cell lines mutated for BRCA2 are sensitive to agents that cause DNA double-stranded breaks and have recombination deficiencies. Using a yeast two-hybrid based genetic screen, I will isolate Rad51 mutants that are defective in interaction with BRCA2 and Rad54. These Rad51 mutants will be characterized biochemically and genetically to establish the significance of the noted protein-protein interactions in recombination and repair. The results from my proposed studies will be important for understanding the mechanistic underpinnings of the human recombination machinery and will make an important contribution toward further delineating the role of recombinational DNA repair in breast tumor suppression.

14. SUBJECT TERMS
recombination, cancer, DNA repair, Rad51, BRCA2

15. NUMBER OF PAGES
7

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Homologous recombination (HR) represents an important means for the repair of chromosome breaks induced by exogenous agents (e.g. ionizing radiation) or that arise endogenously (e.g. replication of a damaged DNA template). The elimination of these breaks is critical for the maintenance of genome stability. DNA strand break repair by HR is mediated by genes of the *RAD52* epistasis group, whose structure and function are highly conserved among eukaryotes (Symington 2002; Sung, Krejci et al. 2003). Mutations in the BRCA2 (breast cancer susceptibility gene 2) gene give rise to a significant portion of familial breast cancers. BRCA2 associates with Rad51, a key member of the *RAD52* group, through a series of conserved modules called BRC repeats. Rad51 has a recombinase activity that mediates the formation of DNA joints between the injured chromosome and an intact homologue during HR. In mediating HR reactions, Rad51 nucleates onto single-stranded (ss) DNA derived from lesion processing to form a highly ordered nucleoprotein filament structure, often referred to as the presynaptic filament. After locating a DNA homologue, the presynaptic filament catalyzes pairing with the homologue to form a nascent DNA joint called a D-loop, the length of which is extended by DNA strand exchange. Concurrently, DNA synthesis commences to replace the genetic information lost during the lesion processing step. Subsequent steps include resolution of recombination intermediates and ligation to complete the recombination/repair reaction. Several comprehensive reviews on this subject have appeared recently (Symington 2002; Sung, Krejci et al. 2003; West 2003). The molecular function of BRCA2 in homologous recombination has not been elucidated and represents the subject of my research project.

Body

Special Note: My host laboratory relocated from the University of Texas Health Science Center at San Antonio to Yale University at the very beginning of the initial budget period. The relocation resulted in substantial disruption of my research activities for almost six months. In spite of this, significant progress has been made on isolating Rad51 mutants that are defective in interaction with the BRC3 repeat of human BRCA2 protein and the portion of human BRCA2 encoded within exon 27. I have also expressed and purified the DNA binding domain of the human BRCA2 protein as well as the BRCA-interacting protein DSS1. A summary of the research progress is provided below.

Key Research Accomplishments

Two-hybrid based mutagenesis to isolate interaction defective mutants

As stated in my original proposal, I have performed two-hybrid analyses to verify the published homotypic interaction of hRad51(Schild, Lio et al. 2000), as well as the heterotypic association of hRad51 with hRad54 (Schild, Lio et al. 2000; Tanaka, Hiramoto et al. 2000) and the BRC repeat of BRCA2 (Wong, Pero et al. 1997; Chen, Chen et al. 1998). Importantly, we have also cloned exon 27 of BRCA2 into the two-hybrid vector, detected interaction with hRad51, and have included it in our mutant screen.

The procedure used for obtaining interaction defective mutants is summarized in Figure 1. Briefly, the hRad51 plasmid used in two-hybrid analysis of protein-protein interactions was passaged through the *E. coli* strain GM4708, which harbors the *mutD5* mutation that inactivates the 3' to 5' exonuclease proofreading activity of DNA polymerase III and thus renders this strain highly mutagenic. Four pools of DNA with increasing levels of acquired mutations, based on the time of amplification of plasmid DNA in rich media, have been obtained. An initial screen of the mutagenized plasmid pools have led to the identification of several candidates that have lost the ability to interact with the BRC3 repeat and with the C-terminal portion of BRCA2 encoded by exon 27. Furthermore, we have carried out a truncation analysis of hRad51, which revealed that the N-terminus of this recombinase is dispensable for the interaction with exon 27 of BRCA2 while being indispensable for interactions with BRC3 and hRad54.

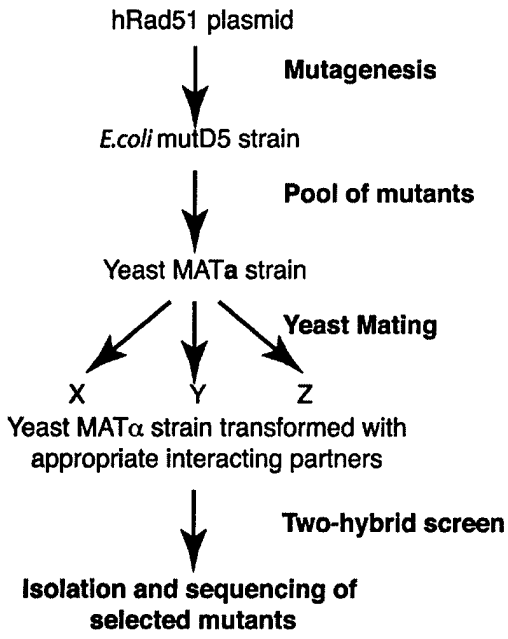

Figure 1. Mutant isolation. Schematic of the mutagenic screen, as per (Krejci, Damborsky et al. 2001). The hRad51 protein on the plasmid is subjected to *in vivo* mutagenesis in E. coli *mutD5*. The mutagenized plasmid collection is introduced into a *MATa* tester yeast strain, which is then mated to a series *MATa* strains containing the interacting partners in plasmids used for two-hybrid interaction. Variants of the mutagenized hRad51 protein that fail to bind one or more of its partners are then selected as clones that fail to grow on the appropriate omission media because of the lack of two-hybrid interaction.

Expression and purification of functional domains of BRCA2

(i) The BRCA2 C-terminal Rad51 binding domain – The portion of BRCA2 protein encoded within Exon 27 that contains a Rad51 binding domain has been amplified by PCR and tailored into an E. coli expression vector that expresses this domain as a fusion to GST. The GST-BRCA2 Exon 27 fusion protein is soluble and has been partially purified by affinity chromatography on Glutathione Sepharose (Fig. 2B). I am in the process of devising a procedure for separating the full-length fusion protein from the proteolytic products. Initial characterization by a pulldown assay (through the GST tag) has found that the GST-BRCA2 Exon 27 fusion protein has the ability to bind hRad51. This fusion protein construct will be valuable for the biochemical testing of mutations that inactivate the two hybrid interaction between the BRCA2 C-terminus and hRad51.

(ii) Expression and purification of human BRCA2 DNA binding domain (BRCA2-DBD) - In the mouse BRCA2 orthologue, from residues 2378 to 3114, three OB folds, a helical domain, and a Tower domain are found; this portion of mouse BRCA2 is called the DNA binding domain (Brca2-DBD). A binding site for the DSS1 extends from the helical domain to OB1. In a DNA binding assay, the purified mouse Brca2-DBD/DSS1 complex binds oligo-dT, forming several distinct nucleoprotein complexes. A co-crystal of the DBD (minus the Tower domain)/DSS1 complex and oligo dT(9) shows contacts of the bases and phosphodiester backbone in the DNA

by aromatic and basic residues in OB2 and OB3. The mouse DSS1 protein was found to be critical for the stability of the mouse Brca2-DBD (Yang, Jeffrey et al. 2002). By PCR amplification, the equivalent segment of human BRCA2 that encodes the DBD domain has been cloned and tailored into an E. coli protein expression vector. Importantly, the human BRCA2-DBD is stable in the absence of DSS1 and soluble. A procedure has already been devised to purify the human BRCA2-DBD to near homogeneity (Fig. 2B).

(iii) Expression and purification of human DSS1 protein – The human DSS1 gene (courtesy of Alan Ashworth, Cancer Research UK) was tailored into an E. coli protein expression vector to express DSS1 as a fusion to GST. In the GST-DSS1 construct, the GST portion can be cleaved with the PreScission protease. The GST-DSS1 fusion protein is soluble, and a procedure has been devised to purify it to near homogeneity (Fig. 2C).

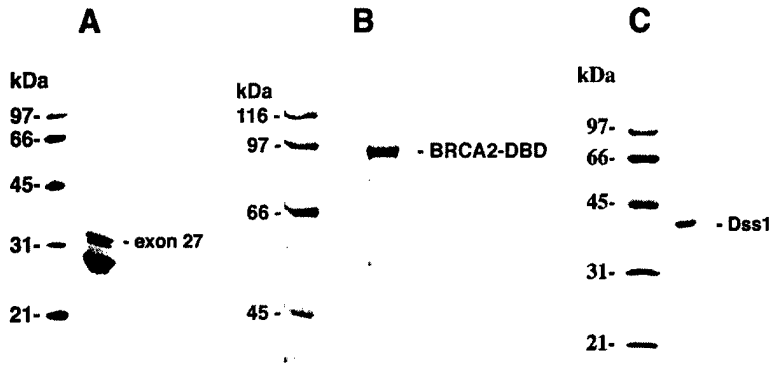

Figure 2. Purified human proteins. Individual proteins were analyzed by SDS-PAGE and staining with Coomassie Blue, including GST-BRCA2 Exon 27 in **(A)**, BRCA2-DBD in **(B)**, and GST-Dss1 in **(C)**.

Future Plan

The two-hybrid Rad51 mutant clones that appear to be impaired for BRC3 and BRCA2-Exon 27 interactions will be sequenced and the mutations will be tailored into our E. coli expression vector for the purification of the mutant proteins. These mutant hRad51 proteins will be examined for all the known hRad51 activities and tested for in vitro interaction with purified GST-BRC3 that we have available. The two-hybrid mutant screen will be extended to isolate hRad51 mutant variants that are defective in hRad54 binding; these mutants will be similarly purified and examined. I am in the process of verifying that **(i)** the GST-BRCA2 Exon 27 fusion protein has hRad51 binding ability, **(ii)** the purified human BRCA2-DBD binds DNA, and **(iii)** the purified human DSS1 protein forms a complex with the purified human BRCA2-DBD. Once goals **(ii)** and **(iii)** have been accomplished, I will begin addressing whether DSS1 influences the DNA binding ability of the BRCA2-DBD.

Conclusions

Even though the link of BRCA2 to homologous recombination and DNA repair is now well established, it remains mysterious at the mechanistic level how this protein fulfills its biological mission. In this regard, my research studies should yield insights into the action mechanism of BRCA2 in homologous recombination and lead to better understanding of the basic mechanism of homologous recombination in human cells. The molecular information garnered from my studies could very well provide the basis for devising new strategies for the prevention and treatment of familial breast cancer.

Reportable Outcomes

Krejci, L., Van Komen, S., Li, Y., Villemain, J., Reddy, M. S., Klein, H., Ellenberger, T., Sung, P. (2003) DNA helicase Srs2 disrupts the Rad51 presynaptic filament. ***Nature*** 423:305-9.
Featured in Nature News & Views "Disruptive Influence" by Marco Foiani, (2003), 423:234-235.

Sung, P., **Krejci, L.**, Van Komen, S. & Sehorn, M. G. (2003) Rad51 recombinase and recombination mediators. ***J. Biol. Chem.*** 278:42729-32.

Van Komen, S., Reddy, M. S., **Krejci, L.**, Klein, H. & Sung, P. (2003) ATPase and DNA helicase activities of the Saccharomyces cerevisiae anti-recombinase Srs2. ***J. Biol. Chem.*** 278:44331-7.

Krejci, L., Chen, L., Van Komen, S., Sung, P., and Tomkinson, A., (2003) Mending the break: the two repair machines in eukaryotes. ***Prog. Nucl. Acid Res. & Mol. Biol.*** 74:159-201.

Krejci, L., Van Komen, S., Macris, M., Villemain, J., Li, Y.,Klein, H., Ellenberger, T., Sung, P. (2004) Role of ATP hydrolysis in the anti-recombinase function of *Saccharomyces cerevisiae* Srs2 protein. ***J. Biol. Chem.*** 279:23193-9.

References Cited

Chen, P. L., C. F. Chen, et al. (1998). "The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment." Proc Natl Acad Sci U S A **95**(9): 5287-92.

Krejci, L., J. Damborsky, et al. (2001). "Molecular dissection of interactions between Rad51 and members of the recombination-repair group." Mol Cell Biol **21**(3): 966-76.

Schild, D., Y. C. Lio, et al. (2000). "Evidence for simultaneous protein interactions between human Rad51 paralogs." J Biol Chem **275**(22): 16443-9.

Sung, P., L. Krejci, et al. (2003). "Rad51 recombinase and recombination mediators." J Biol Chem **278**(44): 42729-32.

Symington, L. S. (2002). "Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair." Microbiol Mol Biol Rev **66**(4): 630-70, table of contents.

Tanaka, K., T. Hiramoto, et al. (2000). "A novel human rad54 homologue, Rad54B, associates with Rad51." J Biol Chem **275**(34): 26316-21.

West, S. C. (2003). "Molecular views of recombination proteins and their control." Nat Rev Mol Cell Biol **4**(6): 435-45.

Wong, A. K., R. Pero, et al. (1997). "RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2." J Biol Chem **272**(51): 31941-4.

Yang, H., P. D. Jeffrey, et al. (2002). "BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure." Science **297**(5588): 1837-48.